

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 7/02, A61K 37/02	A1	(11) International Publication Number: WO 92/20707 (43) International Publication Date: 26 November 1992 (26.11.92)
(21) International Application Number: PCT/US92/03287 (22) International Filing Date: 21 April 1992 (21.04.92) (30) Priority data: 704,863 23 May 1991 (23.05.91) US (71) Applicant: MERRELL DOW PHARMACEUTICALS INC. [US/US]; 2110 East Galbraith Road, P.O. Box 156300, Cincinnati, OH 45215-6300 (US). (72) Inventors: EDWARDS, Judson, V. ; 1086 Lanny Lane, Cincinnati, OH 45231 (US). FANGER, Bradford, O. ; 5019 Cooper Road, Apartment 4, Cincinnati, OH 45242 (US).		(74) Agents: COLLIER, Kenneth, J. et al.; Marion Merrell Dow Inc., 2110 East Galbraith Road, P.O. Box 156300, Cincinnati, OH 45215-6300 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), NO, SE (European patent). Published <i>With international search report.</i>
(54) Title: BOMBESIN ANALOGS (57) Abstract <p>Disclosed are Agonists and Antagonist of bombesin which are derivatives of naturally occurring bombesin possessing a methyl sulfide or a methyl amide bond connecting the two amino acids on the carboxy terminal end. Agonist and antagonist activities are confirmed using conventional competitive binding and biochemical assays as well as conventional physiological tests and the use of these derivatives in a variety of conditions. Use of these peptides include stimulating or antagonizing growth of tissues, especially lung, and a means for effecting treatment for digestional disorders. Treatment comprises administering to a patient in need thereof, an effective amount of a bombesin analog.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MI	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NI	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MC	Madagascar		
ES	Spain				

BOMBESIN ANALOGSFIELD OF INVENTION

5 This invention relates to novel Bombesin analogs to which are potentially useful as pharmaceuticals.

BACKGROUND OF INVENTION

10 Bombesin (ID#2) is a 14 amino acid peptide, originally isolated from the skin of the frog Bombina bombina. Bombesin is also structurally related to a number of other peptides including Gastrin Releasing Peptide (ID#1), and
15 Litorin (ID#3) (See Sequence Identification Section).

Bombesin is known to have a range of effects including stimulation of the nervous system, reduction of renal blood flow, secretion of pituitary hormones, growth promotion,
20 memory retention, induction of myoelectric and contractile activity of intestinal myocytes, induction of gastric and pancreatic secretion, and bolstering of immune function. There has been considerable interest modulating these activities in the design and development of bombesin analogs
25 as possible mimics or inhibitors of bombesin action in the body.

SUBSTITUTE SHEET

The bombesin-dependent responses occur through a class of high-affinity ($KD=1nM$) cell surface receptors that bind bombesin. Binding of Bombesin to its cell surface receptor elicits cell mitogenic responses in a number of tissues. The
5 mitogenic response has been demonstrated in a number of cell types including Swiss 3T3 embryo fibroblast cells, human bronchial epithelial cells, human small cell lung carcinoma cells, rat gastrin cells, and rat pancreatic cells. Similarly, Bombesin induction of gastric and pancreatic
10 secretions, important for digestive functions, occur through the receptors found on cells of pancreatic (B-Cells) and intestinal gastrin cells (G-cells).

Binding of Bombesin to its extracellular receptor evokes
15 a number of intracellular signals including activation of G-proteins, which in turn activates phospholipase C (PLC). PLC in turn converts phosphatidylinositol phosphate (PI) into inositol 1,4,5,-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG are believed to be intracellular signals
20 for cellular mediated events.

Structure-activity studies indicate that receptor-binding requires a peptide ligand containing an amidated C-terminal, and generally the presence of the last eight amino
25 acids. Recent work has concentrated on modifying the carboxy terminal (C-terminal) region of Bombesin to selectively modulated the receptor interaction utilizing a variety of different types of C-terminal modified analogs. These modifications have included, for example,
30 incorporation of D-amino acids, non-peptide bonds, amide, and ester modifications. These alterations have given rise to certain peptides having improved characteristics.

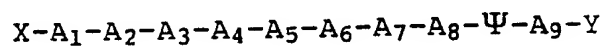
The applicants have prepared linear peptide analogs of
35 the natural bombesin containing a non-peptide bond between

amino acids 8 and 9, consisting of a methyl sulfide group ($\Psi[\text{CH}_2\text{S}(\text{CH}_3)]$) or a methyl amide group ($\Psi[\text{CH}_2\text{N}(\text{CH}_3)]$). The applicants have demonstrated that these analogs act at the bombesin receptor and elicit or prevent required intracellular signals for cellular response of bombesin. The peptide analogs of this invention potentially possess significant mitotic and/or secretory activity and therefore may allow for a scientifically interesting and therapeutically significant adjunct to growth therapy and/or the treatment of digestive disorders. Moreover, the presence of methyl sulfide and methyl amide functionalities, or des-methionine analogs having D-amino acids and N-terminal modifications may provide for enhanced potency and extended duration of action.

15

SUMMARY OF THE INVENTION

20 Claimed are peptide derivatives of the formula 1 given below:



25 wherein X is an amino terminal residue selected from hydrogen, one or two alkyl groups from 1 to 16 carbon atoms, one or two acyl groups of from 2 to 16 carbon atoms, carbobenzyloxy or t-butyloxy carbonyl; unless the amino terminal amino acid is a cyclic derivative and thereby X is omitted.

30 A_1 is pGlu, Glu, or suitable acidic hydrophilic amino acid residue or is a sequence of 1 to 5 amino acids of Bombesin or a natural variants thereof, or a bond;

35

- 5 A₂ is Gln, or suitable neutral amino acid residue;
 A₃ is Trp, or a suitable neutral or hydrophobic
 amino acid residue;
 A₄ is Ala, or a suitable neutral or hydrophobic
 amino acid residue;
 A₅ is Val, or a suitable neutral or hydrophobic
 amino acid residue;
 A₆ is Gly, Ala, ala, or a suitable neutral or
 hydrophobic amino acid residue;
10 A₇ is His, a suitable neutral or basic hydrophilic
 amino acid residue;
 A₈ is Phe, Leu, or is a suitable hydrophobic amino
 acid residue;
 Ψ is a dipeptide determinant of A₈ΨA₉ wherein Ψ
15 is [CH₂S(CH₃)] or [CH₂N(CH₃)], and wherein A₈
 and A₉ designates the substituent amino acids;
 A₉ is Leu, Met, Nle or is a suitable hydrophobic
 amino acid residue or is a sequence of 1 to 5
 amino acid residues of Bombesin or variants
20 thereof, or a bond; and
 Y is a carboxy terminal substituent of the
 carbonyl group of the A₉ amino acid selected
 from OH, (C₁-C₈) alkoxyester, carboxamide, mono
 or di (C₁-C₈) alkyl amide, (C₁-C₈) alkylamine,
25 (C₁-C₄) thioalkylether, or pharmaceutically
 acceptable salt thereof.
- 30
- 35

DETAILED DESCRIPTION OF THE INVENTION

5

The following common abbreviations of; (1) amino acids and their three letter codes, and (2) terminal amino and carboxy substituents used throughout this specification:

10 (1): THE AMINO ACIDS AND THEIR THREE LETTER CODE

	<u>L-AMINO ACIDS</u>	<u>D-AMINO ACIDS</u>
	Ala - alanine	ala - D-alanine
	Arg - arginine	arg - D-arginine
15	Asn - asparagine	asn - D-asparagine acid
	Cys - cysteine	cys - D-cysteine
	Gly - glycine	
	Glu - glutamic acid	glu - D-glutamic acid
20	Val - valine	val - D-valine
	Gln - glutamine	gln - D-glutamine
	His - histidine	his - D-histidine
	Ile - isoleucine	ile - D-isoleucine
	Leu - leucine	leu - D-leucine
25	Lys - lysine	lys - D-lysine
	Phe - phenylalanine	phe - D-phenylalanine
	Met - methionine	met - D-methionine
	Pro - proline	pro - D-proline
	Ser - serine	ser - D-serine
30	Thr - threonine	thr - D-threonine
	Trp - tryptophan	trp - D-tryptophan
	Tyr - tyrosine	tyr - D-tyrosine
	Nle - norleucine	

35

(2): AMINO AND CARBOXY TERMINAL ACID SUSTITUENTS

	Ac	- acetyl
5	Azt	- azetidine-2-carboxylate
	Cin	- cinnamoyl
	DhCin	- 3,4-dihydrocinnamoyl
	Glt	- glutaryl
	Mal	- maleyl
10	Oac	- 8-aminooctanoic acid
	Oct	- n-octane
	Suc	- succinyl
	Glt	- glutaryl
	Tfa	- trifluoroacetyl
15	#	- C-terminal amide

As many as 13 bombesin-like peptides have been isolated from amphibian sources, one from avian proventriculus, and 5 or 6 from mammalian tissues. The Bombesin peptides may be
20 divided into 3 subfamilies, on the basis of their primary structure, their pharmacological activity, and their receptor affinity. The bombesin subfamily is characterized by the C-terminal tetrapeptide -Gly-His-Leu-Met-NH₂, the litorin/ranatensin subfamily by the tetrapeptide -Gly-His-
25 Phe-Met-NH₂, and the phyllolitorin subfamily by the tetrapeptide -Gly-Ser-Phe(Leu)-Met-NH₂.

Present within the bombesin subfamily are the gastrin-releasing peptides (GRPs) of mammalian origin. Human,
30 porcine, and canine GRPs differ from each other in the N-terminal dodecapeptide, but have an identical carboxy amino acid sequences (residues 13-27). Moreover, the C-terminal decapeptide of the mammalian GRPs are identical to the C-terminal decapeptide of frog bombesin, with only the
35 difference of having a His residue substituted for the Gln

residue at position 8 from the C-terminus. A mammalian peptide present within the litorin/ranatensin-like family is neuromedin B.

5 A Sequence Identification of some of the sequence variations of Bombesin is included prior to the claims: e.g. Bombesin (ID#2), Gastrin Releasing Peptide(ID#1), Litorin(ID#3).

10 Herein, the term "bombesin or natural variant thereof" includes all subfamilies and natural variants of bombesin (ID#2)[See Falconieri, et.al. Regulatory Peptides, 21, 1-11, 3, (1988), for a listing of known Bombesin related peptides and is incorporated herein by reference] including sequences
15 related to GRP (ID#1), and Litorin(ID#3) and the like. The term "variations thereof" for substituents A₁ and A₉ optionally includes 1-5 amino acids of bombesin or related variants contiguous with a consecutive region of the amino acids A₂ to A₈ as defined; unless it is a bond or unless the
20 amino or carboxy terminal acid is a cyclic derivative and thereby the sequence of 1-5 amino acids is omitted.

25

30

35

Amino Acids & Modifications

Herein, as is customary, the structure of peptides when written is such that the amino terminal end appears on the left side of the page and the carboxy terminal end appears on the right side of the page.

An alkyl group of 1-8 carbon atoms and the alkyl portion of an alkoxy group is taken to include straight, branched, or cyclic alkyl groups, for example, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, pentyl, isopentyl, sec-pentyl, cyclopentyl, hexyl, isohexyl, cyclohexyl and cyclopentylmethyl, heptyl, octyl(Oct), 8-aminooctanoic acid(Aoc). An acyl group of from 2 to 8 carbon atoms is taken to include straight, branched, cyclic, saturated and unsaturated acyl groups having 1 or 2 carbonyl moieties per group, for example, acetyl(Ac), azetidine-2-carboxylate(Azt), benzoyl, succinyl, cinnamoyl(Cin), 3,4-dihydrocinnamoyl(DhCin), maleyl(Mal), palmityl, lauryl, octanoyl, and glutaryl(Glt). Both alkyl and acyl substituents are taken to include those groups with halogen substituents, where a halogen group is a fluoro, chloro, bromo or iodo, for example, trifluoroacetyl(Tfa). Internally cyclized derivatives of N-terminal amino acid residues include pyroglutamic acid (pGlu) and homoserine lactone (Hse). Presence of a internally cyclized amino acid involving the N-amino group serves to terminate the peptide chain, thereby limiting the extension of the peptide chain and the presence of chemical substituents on the N-amino group.

The naturally occurring amino acids, with the exception of glycine, contain a chiral carbon atom. Unless otherwise specifically indicated, the optically active amino acids,

referred to herein, are of the L-configuration. However, any of the amino acids of the A₁ or A₉ group can be specifically designated to be either the of the D- or L-configuration.

5

The amino acids of A₁ through A₉ essentially consists of the naturally occurring amino acids which are glycine, alanine, valine, leucine, isoleucine, serine, methionine, threonine, phenylalanine, tyrosine, tryptophan, cysteine, 10 proline, histidine, aspartic acid, asparagine, glutamic acid, glutamine, arginine, ornithine, and lysine. Also included would be the D-isomers of the naturally occurring amino acids; D-alanine, D-valine, D-leucine, D-isoleucine, D-serine, D-methionine, D-threonine, D-phenylalanine, D- 15 tyrosine, D-tryptophan, D-cysteine, D-proline, D-histidine, aspartic acid, D-asparagine, D-glutamic acid, D-glutamine, D-arginine. As indicated earlier, D amino acids may be represented by the first letter of their 3 letter or 1 letter code being a lower case letter; i.e for D-Alanine 20 (ala, or a).

Groups of amino acids can be defined by certain charge characteristics. There are two general characteristics of side chains: nonpolar and polar. The nonpolar residues are 25 made up of these groups: the hydrophobic residues which includes those with (1) aliphatic hydrocarbon side chains: Gly, Ala, Val, Leu, Ile, Nle, Pro and (2) the aromatic group Phe and Trp, and (3) the pseudohydrocarbon, Met. The polar amino acids are made up three groups: (1) The acidic 30 hydrophobic residues Asp, Glu, and Tyr, (2) the neutral residues with the hydroxyl-containing residues, Ser and Thr; the amides, Asn and Gln; the aromatic rings, Tyr and Trp; the sulfhydryl groups, Cys, and small structurally accommodating amino acids Ala and Gly, and (3) basic 35 hydrophobic residues His, Lys, and Arg.

Y designates the chemical group(s) that may be utilized to substitute or modify the terminal amino acid. Therefore, Y may be a carboxy terminal acid (-OH), C₁-C₈ alkoxyester, 5 carboxamide, mono or di C₁-C₈ alkylester, C₁-C₈ alkylamine, or C₁-C₄ thioalkylether, or a pharmaceutically acceptable salt in addition or in conjunction with any of the substituents.

10 The polypeptides of formula 1 can form pharmaceutically acceptable salts with any non-toxic, organic or inorganic acid. Illustrative inorganic acids which form suitable salts include hydrochloric, hydrobromic, sulphuric and phosphoric acid and acid metal salts such as sodium
15 monohydrogen orthophosphate and potassium hydrogen sulfate. Illustrative organic acids which form suitable salts include the mono, di and tricarboxylic acids. Illustrative of such acids are, for example, acetic, glycolic, lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric,
20 citric, ascorbic, maleic, hydroxymaleic, benzoic, hydroxybenzoic, phenylacetic, cinnamic, salicylic, 2-phenoxybenzoic and sulfonic acids such as methane sulfonic acid and 2-hydroxyethane sulfonic acid. Salts of the carboxy terminal amino acid moiety include the non-toxic
25 carboxylic acid salts formed with any suitable inorganic or organic bases. Illustratively, these salts include those of alkali metals, as for example, sodium and potassium; alkaline earth metals, such as calcium and magnesium; light metals of Group IIIA including aluminum; and organic
30 primary, secondary and tertiary amines, as for example, trialkylamines, including triethylamine, procaine, dibenzylamine, 1-ethenamine, N,N'-dibenzylethylenediamine, dihydroabietylamine, N-(lower)alkylpiperidine, and any other suitable amine.

35

-11-

It is understood amino acids containing the methyl sulfides and the methyl amides existent herein are designated ($\Psi[\text{CH}_2\text{S}(\text{CH}_3)]$) and ($\Psi[\text{CH}_2\text{N}(\text{CH}_3)]$) respectively. Utilizing conventional nomenclature employed by peptide chemists, $\text{A}_8\text{-}\Psi\text{-A}_9$ are those compounds wherein the moiety connecting the two amino acids A_8 and A_9 is by a modified peptide linkage; such as by a methylene methyl sulfide or methylene methyl amide bond. For example, where the A_8 residue is Phe linked to the A_9 Leu residue by a methylene methyl sulfide or methylene methyl amide bond, they can be designated respectfully as $\text{Phe}\Psi[\text{CH}_2\text{S}(\text{CH}_3)]\text{Leu}$ and $\text{Phe}\Psi[\text{CH}_2\text{N}(\text{CH}_3)]\text{Leu}$. This designation indicates that the carbonyl group of the penultimate Phe is reduced to a methylene which is bonded to the methyl sulfide group or methyl amide group of The A_9 substituent respectively.

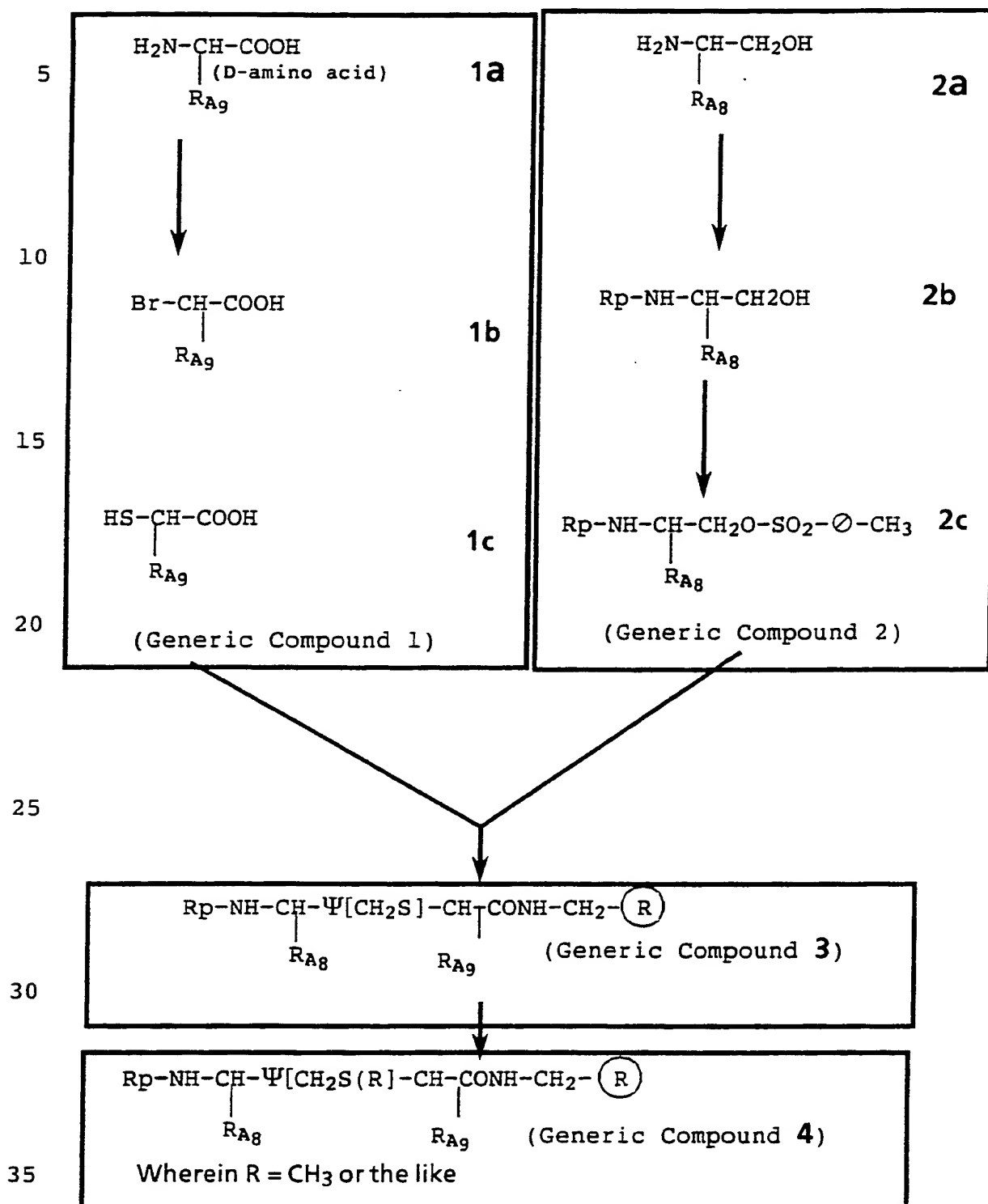
The procedure to prepare starting materials of formula 1 wherein Ψ is a $-\text{CH}_2\text{S}(\text{CH}_3)-$ group, that is the $\Psi[\text{CH}_2\text{S}]$ compounds, is known from Spatola, A.F. and Edwards, J.V., Biopolymers, 25, S229-S244 (1986), hereby incorporated by reference, and Spatola and Darlak, Tetrahedron Letters, 44(3), 821-33 (1986), hereby incorporated by reference. Similarly the procedure to prepare starting materials of formula 1 wherein Ψ is a $-\text{CH}_2\text{N}(\text{CH}_3)-$ group, that is the $\Psi[\text{CH}_2\text{N}(\text{CH}_3)]$ compounds, is known from Sasaki and Coy, Peptides 8, 119-121 (1986), hereby incorporated by reference.

Synthesis of compounds having modified dipeptide substituents of the structure $\text{A}_8\text{-}\Psi[\text{CH}_2\text{S}(\text{CH}_3)]\text{-A}_9$ can be obtained (scheme 1). The modified dipeptides of scheme 1 are obtained by initially preparing the modified amino acids shown as Generic Compound 1 and Generic Compound 2.

35

SUBSTITUTE SHEET

REACTION SCHEME 1



The Generic Compound 1 is obtained by starting with a D-amino acid having a R_{A9} group (1a). R_{A9} designates structures of desired amino acid of 1a when taken as a substituent with the α -amino acid. Suitable protection of reactive groups present on the R_{A9} substituent can optionally be selected. Such selection for R_{A9} protection is described in the literature and are well known to those skilled in the art. To synthesize Generic Compound 1, the D-amino acid 1a is first halogenated to produce the α -halo R_{A9} substituted acid 1b. The α -bromo R_{A9} substituted acid 1b can suitably be formed using potassium bromide in aqueous sulfuric acid. The α -halo R_{A9} substituted acid 1b can then be converted to the α -mercapto R_{A9} acid by treatment with salts of mercaptans (e.g. thiolate ions). A suitable method for forming α -mercapto R_{A9} acid is reaction with sodium trithocarbonate, followed by workup of the reaction product to afford the α -mercapto, R_{A9} -alkanoic acid 1c.

Generic Compound 2 can be obtained by starting with an L- α -amino R_{A8} -substituted alcohol 2a. The α -amino group can then be suitable protected for peptide synthesis, as is well known in the art. Suitable protection can be afforded by the di-*t*-butyloxycarbonate (Boc) protecting group to form, for example, the Boc-amino- R_{A8} -substituted alcohol 2b, where it is understood Boc is a suitable substituent for Rp. The alcohol functionality of 2b can then be activated to a suitable leaving group, as in 2c, for condensation with Generic Compound 1c. The formation of the tosylated ($-\text{SO}_2-\text{O}-\text{CH}_3$) leaving group present in 2c has been found to be suitable for reaction with the generic compound 1 for condensation.

The Generic Compound 3 is obtained by reacting the Generic compound 1c with Generic Compound 2c resulting in

SUBSTITUTE SHEET

the substitution of the sulfide and displacement of the tosylate group. This can suitably be done by reacting Generic Compound 1c with sodium ethoxide to preform the disodium salt of the mercaptoacid and then react the mercaptoacid salt with Generic Compound 2c, displacing the tosyl group to form the dipeptide of Generic Compound 3. Compounds of the structure 3 can be optionally linked to a resin support ($\textcircled{\text{R}}$), by methods known in the art and described herein.

10

Generic Compound 3 then can be conveniently converted to the methyl sulfide of Generic Compound 4. Methylation of the sulfide can be done with a number of methylating reagents. A suitable means of accomplishing this step is to react the generic compound 3 with iodomethane to form the sulfur-ylide for isolation. Methylation of compounds of the structure 4 can be optionally linked to a resin support ($\textcircled{\text{R}}$).

15

Similarly, methylation of the dipeptide linkage can be done before or after synthesis of the desired peptide sequence on the support, however, it is generally preferred to be done after the desired sequence has been completed.

20

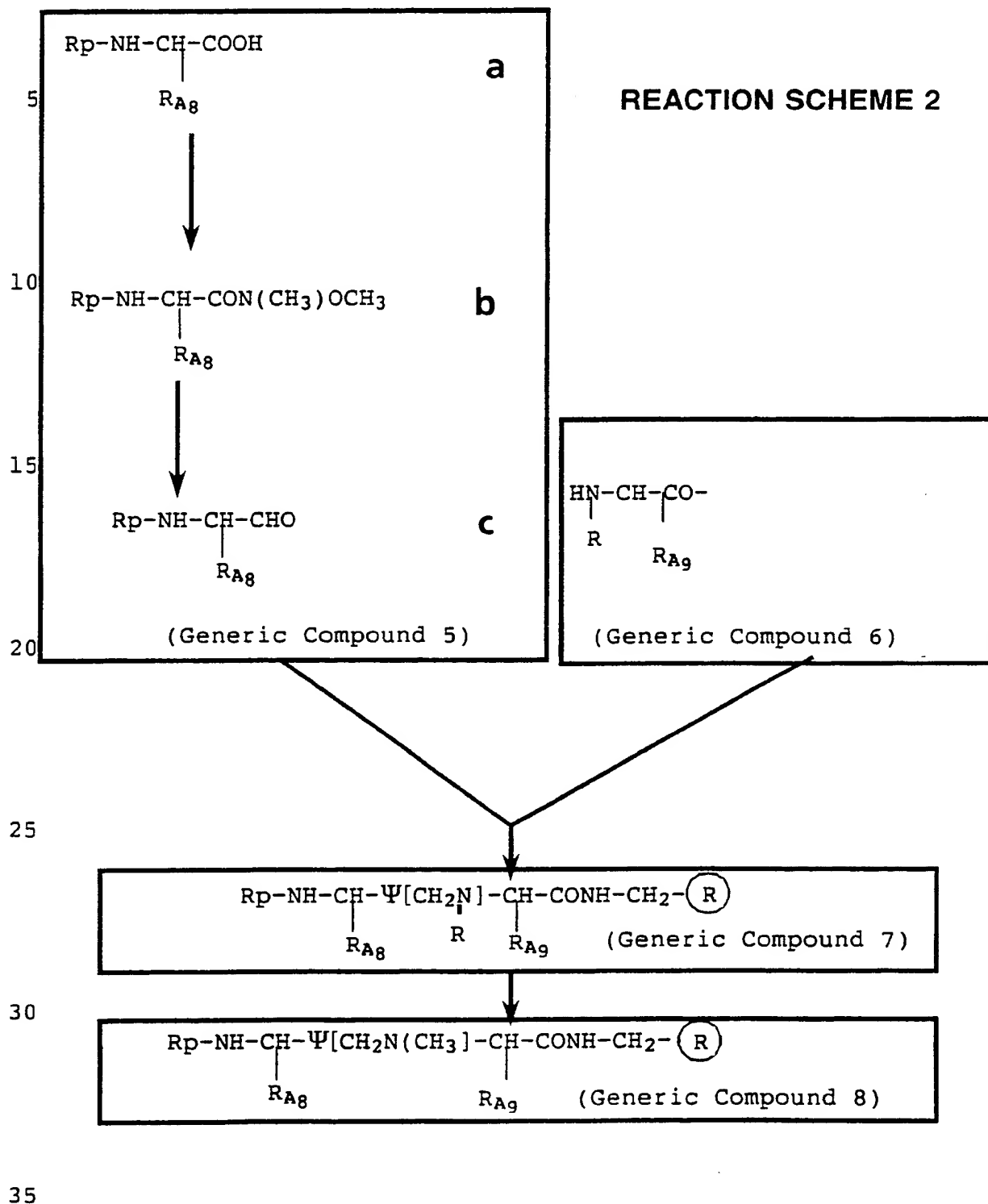
Synthesis of compounds having modified dipeptide substituents of the structure $\text{A}_8\text{-}\Psi[\text{CH}_2\text{N}(\text{CH}_3)]\text{-A}_9$ can be generally be obtained (scheme 2).

25

30

35

REACTION SCHEME 2



SUBSTITUTE SHEET

The modified dipeptides of scheme 2 are obtained by initially preparing the modified amino acids shown as Generic Compound 5 and Generic Compound 6.

5 α -(Acylamino) and α -(alkoxycarbonylamino) aldehydes of Generic Compound 5c can be prepared by oxidation of N-protected amino alcohols or by reduction of amino acids or their esters 5b with diisobutylaluminum hydride. For example, suitably the α -(t-butoxycarbonylamino)-aldehydes
10 can be prepared from the corresponding N-methoxy-N-methylcarboxamides by reduction with lithium aluminum hydride when Rp is designated as CH₃ or the like. The N-methoxy-N-methylamides can be prepared by reaction of the α -(t-butoxycarbonylamino) acids with O,N-dimethylhydroxylamine
15 hydrochloride in the presence of triethylamine and the coupling reagent benzotriazol-1-yloxytris(dimethylamino-phosphonium hexafluorophosphate (BOP). Reduction of 5b with lithium aluminum hydride gives the lithium salt of of compound 5c.

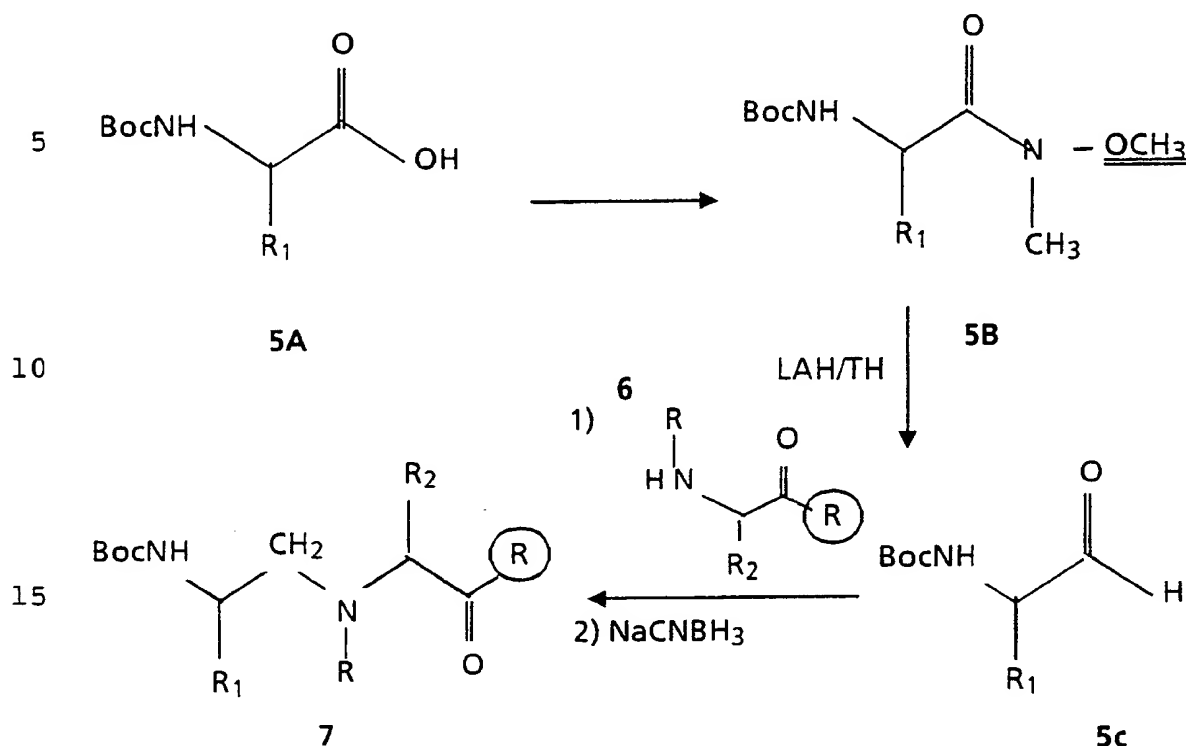
20

Compound 5 (optionally bound to a support resin) and Compound 6 can be reacted in aqueous solution to form the Schiff base between the amine and the aldehyde, which can be subsequently reduced. Suitable reduction of the Schiff base
25 can be carried out with sodium borohydride (or derivative thereof) to form the Generic Compound 7. The structure of compound 7 can be suitably methylated as described for Generic Compound 4.

30 Specifically, Compounds of 5c can be prepared by reducing the N-methoxy-N-methylamide of formula 5b to produce the aldehyde of formula 5c.

35

METHOD A

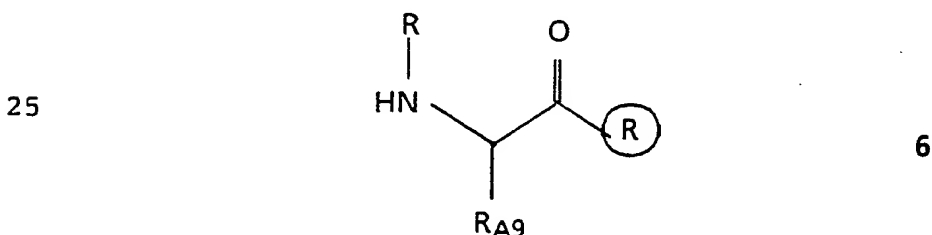


The reduction can be performed in any way generally known and readily performed by those skilled in the art such as by use of lithium aluminum hydride (LiAlH_4). This reduction can be conveniently carried out by adding about one molar equivalent of LiAlH_4 to a cooled, typically about 0°C , solution of a formula 5A compound in a nonreactive solvent such as an ethereal solvent such as tetrahydrofuran (THF) or diethylether. After the reaction is substantially complete, typically after about 30 minutes, the reaction mixture is quenched by the addition of, for example, 10% potassium or sodium hydrogen sulfate and then water. The product 5c can then be isolated by, for example, extraction of the aqueous mixture with a solvent such as diethylether, washing the ether phase with cold, dilute aqueous

hydrochloric acid, drying and solvent removal. The crude product can be purified by, for example, column chromatography such as a silica gel column eluting with 55% ethyl/acetate/hexane.

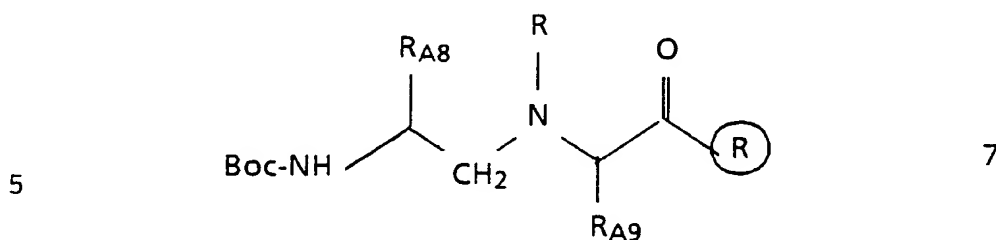
5 The N-methoxy-N-methyl amides of formula 5b can be prepared from the corresponding N-Boc protected acid in the usual manner. Carbonyldiimidazole is added to a dried solution of the N-Boc protected amino acid in an ethereal
10 solvent such as diethylether. The reaction mixture is allowed to stir for from 10 minutes to 1 hour, typically for about 15-20 minutes. N,O-dimethylhydroxylamine HCl in DMF and a sterically hindered amine such as diisopropylethyl amine is added and the mixture allowed to
15 stir for from about 6 hours up to about 24 hours at room temperature. The desired compound is then isolated by solvent evaporation and crude purification can be accomplished by, for example, flash chromatography on silica gel eluting with methylene chloride.

20 The formula 5c aldehyde is then reacted with a resin-bound amino acid of formula 6 to form a Schiff base adduct



wherein R is methyl and RA9 are as defined for formula 1
30 and wherein;

(R) represents the resin. The schiff base adduct r is then reduced in situ, for example, by sodium cyanoborohydride, to give a resin bound modified dipeptide
35 of formula 7



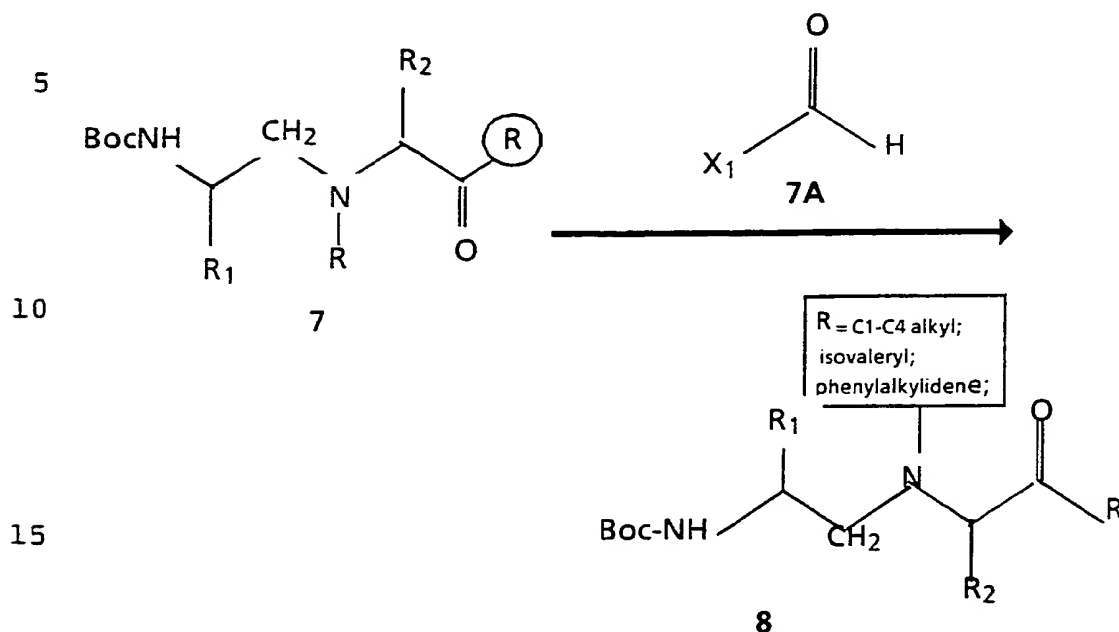
wherein R, RA8 and RA9 are as defined for formula 1 and
 wherein R represents the resin.

Methods of reacting compounds of formula 5c with a
 amino acid of formula 6 on a resin support, through Schiff
 base formation and subsequent reduction to give modified
 dipeptides of formula 7, are preferred when R is hydrogen
 or methyl.

Alternative methods (Method B) of making the compounds
 of formula 5, wherein R is methyl, ethyl, propyl,
 isovaleryl, or like alkyl substituent of 1-5 carbon atoms,
 or phenylalkylidene, can be performed by reductive
 alkylation. Specifically, compounds of formula 7 wherein R
 is hydrogen can be subjected to a subsequent reaction with
 with compounds of the formula 7A to produce the modified
 dipeptide of formula 8, wherein the subsequent R group is
 derived from the substituted alkyl group (represented as X1
 and a functional aldehyde group).

The alternative method (method B) first reacts a
 formula 7A aldehyde with the resin bound dipeptide of
 formula 7, wherein R is a hydrogen group and the circled R
 represents the resin. The initially formed Schiff base
 adduct is then reduced in situ using, for example, sodium
 cyanoborohydride to give a resin bound dipeptide of formula
 8. The A7 through A1 amino acids can then be sequentially

METHOD B



20 added to the resin bound modified dipeptide in the usual manner.

The resin support employed can be any suitable resin conventionally employed in the art for the solid phase preparation of polypeptides, preferably polystyrene which has been cross-linked with from 0.5 to about 3 percent
25 divinyl benzene, which has been either chloromethylated or hydroxymethylated to provide sites for ester formation with the initially introduced α -amino protected amino acid.

An example of a hydroxymethyl resin is described by
30 Bodanszky, et al., Chem. Ind. (London) 38, 1597-98 (1966). A chloromethylated resin is commercially available from Bio Rad Laboratories, Richmond, California, and the preparation of such a resin is described by Stewart and Young, "Solid Phase Peptide Synthesis" (Freeman & Co., San Francisco
35 1969), Chapter 1, pp. 1-6. The protected amino acid can be

SUBSTITUTE SHEET

bound to the resin by the procedure of Gisin, Helv. Chem Acta, 56, 1476 (1973). Many resin bound, protected amino acids are commercially available. As an example, to prepare a polypeptide of this invention wherein the carboxy
5 terminal end is a Thr residue, a tert-butyloxycarbonyl (Boc) protected Thr bound to a benzylated, hydroxy-methylated phenylacetamidomethyl (PAM) resin can be used and is commercially available.

10 Peptide Synthesis:

The peptides of formula 1 of this invention can be prepared by a variety of procedures readily known to those skilled in the art. Such procedures include the solid phase
15 sequential and block synthesis, gene cloning and combinations of these techniques. The solid phase sequential procedure can be performed using established automated methods such as by use of an automated peptide synthesizer. Peptides of formula 1 were synthesized on the
20 resin beginning with a protected dipeptide containing a either a inter-amino acid methylene methyl sulfide or a methylene methyl amide bridge with the C-terminal amino acid of the dipeptide attached to a methylbenzhydrylamine resin. Peptides of formula 2 traditionally have the carboxy
25 terminal amino acid attached to a methylbenzhydrylamine resin for subsequent extension. The extension of the peptide sequence was done using standard methodology and that of the manufacturer and that known by people skilled in the art. Extension of the peptide chain is by coupled
30 amino acids is known for both L and D isomers of amino acids.

After completion of coupling of the sequence either the Boc protecting group was left in place or it was removed and
35 the N-terminal amino group alkylated or acylated using those

methods known in the art. After the desired N-terminus is formed then displacement of the protecting groups and removal of the peptide from the resin is accomplished using a hydrogen fluoride solution, as known in the art.

5

The α -amino protecting group employed with each amino acid introduced into the polypeptide sequence may be any such protecting group known to the art. Among the classes of α -amino protecting groups contemplated are (1) acyl type
10 protecting groups such as: formyl, trifluoroacetyl, phthalyl, toluenesulfonyl (tosyl), benzenesulfonyl, nitrophenylsulfenyl, tritylsulfenyl, o-nitrophenoxycetyl and α -chlorobutyryl; (2) aromatic urethan type protecting groups such as benzyloxycarbonyl and substituted benzyloxycarbonyl,
15 such as p-chlorobenzyloxycarbonyl, p-nitrobenzylcarbonyl, p-bromobenzyloxycarbonyl, p-methoxybenzyloxycarbonyl, 1-(p-biphenyl)-1-methylethoxycarbonyl, α -dimethyl-3,5-dimethoxybenzyloxycarbonyl and benzhydryloxycarbonyl; (3) aliphatic urethan protecting groups such as tert-butyloxy-
20 carbonyl (Boc), diisopropylmethoxycarbonyl, isopropyl-oxy carbonyl, ethoxycarbonyl and allyloxycarbonyl; (4) cycloalkyl urethane type protecting groups such as cyclopentylloxycarbonyl, adamantylloxycarbonyl and cyclohexylloxycarbonyl; (5) thiourethane type protecting groups
25 such as phenylthiocarbonyl; (6) alkyl type protecting groups such as triphenylmethyl (trityl) and benzyl; and (7) trialkylsilane groups such as trimethylsilane. The preferred α -amino protecting group is tert-butyloxycarbonyl (Boc).

30

As is known in the art of solid phase peptide synthesis many of the amino acids bear functionalities requiring protection during synthesis. The use and selection of the appropriate protecting group will depend upon the amino acid
35 to be protected and the presence of other protected amino

acid residues on the peptide. Generally, the selection of such a side chain protecting group requires that it must be one which is not removed by cleavage during cleavage of the protecting group of the α -amino moiety. For example,

5 suitable side chain protecting groups for lysine are benzyloxycarbonyl and substituted benzyloxycarbonyl, said substituent being selected from halo (e.g., chloro, bromo, fluoro) and nitro (e.g., 2-chlorobenzyloxycarbonyl, p-nitrobenzyloxy-carbonyl, 3,4-dichlorobenzyloxycarbonyl),

10 tosyl, t-amylloxycarbonyl, t-butyloxycarbonyl and diisopropylmethoxycarbonyl. The alcoholic hydroxyl group of threonine and serine can be protected with an acetyl, benzoyl, tert-butyl, trityl, benzyl, 2,6-dichlorobenzyl or benzyloxycarbonyl group. The preferred protecting group is

15 benzyl. The selection and use of appropriate protecting groups for each peptide is within the ability of those skilled in the art.

The selection of an appropriate coupling reagent is

20 within the skill of the art. A particularly suitable coupling reagent where the amino acid to be added is Gln, Asn or Arg is N,N'-diisopropylcarbodiimide and 1-hydroxybenzotriazole. The use of these reagents prevents nitrile and lactam formation. Other coupling agents are (1) carbo-

25 diimides (e.g., N,N'-dicyclohexylcarbodiimide and N-ethyl-N'-(γ -dimethylaminopropylcarbodiimide); (2) cyanamides (e.g., N,N-dibenzylcyanamide); (3) ketenimines; (4) isoxazolium salts (e.g., N-ethyl-5-phenyl-isoxazolium-3'-sulfonate; (5) monocyclic nitrogen containing heterocyclic amides of

30 aromatic character containing one through four nitrogens in the ring such as imidazolides, pyrazolides, and 1,2,4-triazolides. Specific heterocyclic amides that are useful include N,N'-carbonyldiimidazole and N,N-carbonyl-di-1,2,4-triazole; (6) alkoxylated acetylene (e.g., ethoxyacetylene);

35 (7) reagents which form a mixed anhydride with the carboxyl

moiety of the amino acid (e.g., ethylchloroformate and isobutylchloroformate) or the symmetrical anhydride of the amino acid to be coupled (e.g., Boc-Ala-O-Ala-Boc), (8) nitrogen containing heterocyclic compounds having a hydroxy group on one ring nitrogen (e.g., N-hydroxyphthalimide, N-hydroxysuccinimide and 1-hydroxybenzotriazole), and (9) diphenyl phosphorylazide. Other activating reagents and their use in peptide coupling are described by Kapoor, J. Pharm. Sci., 59, pp. 1-27 (1970). Applicants prefer the use of the symmetrical anhydride as a coupling reagent for all amino acids except Arg, Asn and Gln.

Each protected amino acid or amino acid sequence is introduced into the solid phase reactor in about a four-fold excess and the coupling is carried out in a medium of dimethylformamide: methylene chloride (1:1) or in dimethylformamide alone or preferably methylene chloride alone. In cases where incomplete coupling occurs, the coupling procedure is repeated before removal of the α -amino protecting group, prior to the coupling of the next amino acid in the solid phase reactor. The success of the coupling reaction at each stage of the synthesis is monitored by the ninhydrin reaction as described by E. Kaiser et al, Analyt. Biochem. 34, 595 (1970).

Following the coupling of the α -amino protected amino acid to the resin support, the protecting group is removed using any suitable procedure such as by using trifluoroacetic acid in methylene chloride, trifluoroacetic acid alone, or HCl in dioxane. The deprotection is carried out at a temperature of between 0°C and room temperature. Other standard cleaving reagents and conditions for removal of specific α -amino protecting groups may be used. After removal of the α -amino protecting group the other amino protected amino acids are coupled step-wise in the desired

order. Alternatively, multiple amino acid groups may be coupled by the solution method prior to coupling with the resin supported amino acid sequence.

5 After the desired amino acid sequence has been obtained, the peptide is removed from the resin. This can be done by hydrolysis such as by treatment of the resin bound polypeptide with an amino acid alcohol and acetic acid in dichloromethane (DCM). Protecting groups can also be
10 removed by other procedures well known in the art. Typically protecting group removal is done after the peptide chain synthesis is complete but the protecting groups can be removed at any other appropriate time. Purification of peptides is principally accomplished through preparative
15 reverse phase high performance liquid chromatography and those techniques known to those skilled in the art.

The ability of the peptide derivatives of this invention to act as agonists or antagonist of Bombesin can be
20 demonstrated by the ability of such peptides to compete with radioiodinated bombesin/GRP for mammalian bombesin/GRP receptors using the method of Buck, et al., Science 226: 987-989, 1984, and by the ability of such compounds to stimulate bombesin induced phosphatidylinositol turnover
25 using the method of Bristow, et al., British J. Pharmacol. 90: 211-21, 1987.

30

35

Therapeutic Use

5 Stimulating/Inhibition of Digestion

Specific pharmacological effects of bombesin analogs to stimulate digestion have been elicited by systemic injection. For example, intravenous injection of bombesin analogs is able to stimulate gastric acid secretion [reviewed in Walsh, J., Annu. Rev. Physiol. 50, 41-63, (1988)]. Both peripheral and central administration of bombesin peptides delays the gastric emptying while also stimulating gastrointestinal smooth muscles in vitro. It has also been demonstrated, for example, exogenous administration of bombesin induces the release of both gastrin and somatostatin in isolated vascularly perfused rat stomachs. Similarly guinea pig antrum longitudinal muscle strips directly stimulate the frequency of spontaneous contractions and direct the contraction of the muscularis mucosae of the colon. However, it is to be noted that these effects may not occur if their administration is to the brain or spinal cord. The applicants use of the peptide to stimulate/inhibit digestion, are therefore, useful when those effects are consistent with the necessary mechanisms of digestion and are consistent with peripheral administration (i.e., not being injected into the brain or spinal cord).

The natural history of peptic ulcer disease is one of recurrent exacerbations and remissions. As a result, ulcerative diseases should be treated as a chronic disorder. Peptic (esophageal, gastric, and duodenal) ulcers occur in areas of the gastrointestinal tract exposed to acid and pepsin. The compounds of the present invention which are antagonist of the bombesin receptor may be useful in the

treatment of gastrointestinal and/or pancreatic ulcers and may be effective in resultant hypersecretions occurring from the pancreas and/or stomach, particularly hydrochloric acid and pepsin. As such compounds of this invention may serve
5 as an appropriate intervention to treat peptic ulcers.

Stimulation/Inhibition of Growth

Binding of Bombesin to its cell surface receptor elicits
10 cell mitogenic responses in a number of tissues. The initial demonstration that the bombesin peptides could function as mitogens was demonstrated on Swiss 3T3 murine embryonal fibroblasts [Rozengurt and Sinnett-Smith, BBRC 140, 379-385 (1983)]. Latter studies by Represa [Represa
15 J.J., et. al. Development 102, 87-96 (1988)] showed that bombesin could reactivate cell division and development in growth-arrested ocular vesicles. Similar increases in the clonal growth rate and colony-forming efficiency were observed by Willey et. al. 1984 for GRP and GRP analogs
20 [Willey, J.C., et al., Exp. Cell Res 153, 245-248 (1984)]. A number of groups have observed the presence of high-affinity receptors for bombesin/GRP in a number of human small cell lung carcinomal cell lines and showed bombesin could elevate levels of thymidine incorporation with
25 peptides added to the media [See Weber et al., J. Clin. Invest 75, 306-309 (1985); Carney, et al., Cancer Res. 47, 821-825, (1987)]. A measurable effect on gastrin cells in the antral mucosa of the rat stomach were noted by [Lehy et. al., Gastroenterology, 84, 914-919 (1983)] following the
30 administration of bombesin. Chronic treatment of the bombesin has also been shown to induce a dose-dependent pancreatic cell hypertrophy (Lhoste et al. 1985a). The applicants use of the peptide to stimulate growth, are therefore, useful when those effects are consistent with the

necessary mechanisms of growth and are consistent with the effects seen with peripheral administration.

Use of bombesin antagonist in cancer therapy is
5 indicated for the treatment of small cell lung carcinomas (SCLC) and prostatic carcinomas and prevention of a variety of other cancer conditions. Those experienced in this field are readily aware of the circumstances requiring cancer therapy.

10

As used herein, the term "tumor tissue" means both benign and malignant tumors or neoplasms and includes melanomas, lymphomas, leukemias, and sarcomas. Illustrative
15 examples of tumor tissues are cutaneous such as malignant melanomas and mycosis fungoides; hematologic tumors such as leukemias, for example, acute lymphoblastic, acute myelocytic, or chronic myelocytic leukemia; lymphomas such as Hodgkin's disease or malignant lymphoma; gynecologic tumors such as ovarian and uterine tumors; urologic tumors
20 such as those of the prostate, bladder, or testis; soft tissue sarcomas, osseous, or non-osseous sarcomas, breast tumors; tumors of the pituitary, thyroid, and adrenal cortex; gastrointestinal tumors such as those of the esophagus, stomach, intestine, and colon; pancreatic and
25 hepatic tumors; laryngeal papillomatoses and lung tumors.

The term "controlling the growth" and the concept of treating a cancer means slowing, interrupting, arresting, or stopping the growth and metastases of a rapidly
30 proliferating tumor in a warm blooded animal; it being understood that treatment in a warm blooded animal does not generally provide a "cure" for the tumor in the sense that necessarily the tumor tissue is destroyed or totally eliminated.

35

Therapeutic Administration

The appropriate dose of a peptide derivative of this invention when used in the treatment of patient in need thereof is from 0.2 mg/kg to 250 mg/kg of patient body weight per day depending on other factors involving the particular patient and the peptide derivative selected. The suitable dose for a particular patient can be readily determined. Preferably from 1 to 4 daily doses would be administered typically with from 5 mg to 100 mg of active compound per dose. The amount of a peptide of this invention required can be readily determined by those skilled in the art.

15

The term "patient" used herein is taken to mean mammals such as primates, including humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice.

20 Although some of the peptide derivatives may survive passage through the gut following oral administration, applicants prefer non-oral administration, for example, subcutaneous, intravenous, intramuscular or intraperitoneal; administration by depot injection; by implant preparation; 25 or by application to the mucous membranes, such as, that of the nose, throat and bronchial tubes, for example, in an aerosol can containing a peptide derivative of this invention in a spray or dry powder form.

30 For parenteral administration the compounds may be administered as injectable dosages of a solution or suspension of the compound in a physiologically acceptable diluent with a pharmaceutical carrier which can be a sterile liquid such as water and oils with or without the addition 35 of a surfactant and other pharmaceutically acceptable

adjuvants. Illustrative of oils which can be employed in these preparations are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general, water, saline, aqueous dextrose and related sugar solutions, ethanol and glycols such as propylene glycol or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

As pharmacologically useful agents, compounds of formula 1 can be administered in various manners to the patient being treated to achieve the desired effects, such that, the compounds can be administered either alone or in combination with one another, or they can be administered. Specifically, compounds of formula 1 may be useful in combination with standard radiological and or chemical treatments in cancer therapy, whereby the compounds are expected to increase the effectiveness of said radiological or chemical treatments existing in the field. As used herein, the term "conjunctive administration" when used in relation to the administration of compounds of formula 1 means the administration of such compound during the time in which the patient requires such need treatment as medically determined.

25

EXAMPLES

This invention is illustrated by the following, nonlimiting examples.

30

EXAMPLE 1

Preparation of $A_8Y[CH_2S(CH_3)]A_9$ Litorin

2-Mercapto-4-Methylpentanoic Acid (Compound 1).

A solution of D-Leucine (5g) and potassium bromide (114g) in 400 ml of 2.5 N H_2SO_4 was cooled to $-5\text{ }^{\circ}\text{C}$ in an ice

salt bath. A cold solution of NaNO_2 (30g/70ml water, 0-50°C) was added dropwise with stirring. The reaction was allowed to proceed for ~14 hours at room temperature. The reaction was then extracted with 75 ml portions of ether 5 three times. The ether extract was dried over anhydrous sodium sulfate. The solution was filtered and the ether was evaporated. The resulting clear oil, 2-bromo-4-methylpentanoic acid (Martin and Greco, (1968) J. Org. Chem. 33, 1275-1276) (18g) was committed to a 250ml solution of 33% 10 sodium trithiocarbonate with stirring at 0°C. The reaction was stirred for 48 hrs and then acidified at 0°C with judicious addition of 10N H_2SO_4 . The acidified solution was then extracted with 75 ml portions of ether three times. The ether extracts were dried over anhydrous sodium sulfate, 15 and subsequently the ether was removed *in vacuo*. The resulting yellowish oil (17g) was vacuum distilled. The final yield was 15.3g of (S)-2-mercapto-4-methylpentanoic acid; b.p. 92-93 (0.75mmHg); $[\alpha]_{\text{D}}^{25} = -23.2$ (c1, MeOH).

20 (S)-(tert-Butyloxycarbonyl)-2-Amino-3-Phenyl-Propanyl-p-Toluenesulfonate (Compound 2).

The starting reagent for the title compound, was synthesized from (S)-(tert-Butyloxycarbonyl)-2-amino-3-Phenyl-Propanol (4.5g, 0.0179moles; prepared from L-phenyl- 25 alaninol (Sigma) and di-tert-butylidicarbonate). The starting reagent was then added to 20 mls of pyridine under anhydrous conditions and chilled to -40°C in a dry ice/acetone bath. To the mixture tosyl chloride was then added (6.9g, 3.6mmol). The reaction mixture was then run at 30 4°C. No effort was made to remove accumulating deposits of pyridinium chloride. Upon termination of the reaction, the pyridine was removed *in vacuo*, and the resulting solid was exacted in ether. The ether extract was dried over anhydrous sodium sulfate, filtered, and the ether removed *in* 35 *vacuo*; yielding 10.5g of a oil. Crystals of the product were

obtained from precipitation of the oil in ethyl acetate and hexane; yielding 9.0g of a white solid; m.p., 109-110°C.

(S)-(S)-tert-Butyloxycarbonyl-PheΨ[CH₂S]Leu-OH (Compound 3).

5 A 0.43 M solution of sodium ethoxide (Solution A) was prepared with freshly cut sodium and anhydrous ethanol. An ethanol solution of Compound 1, (S)-2-mercapto-4-methyl-pentanoic acid (0.72g in 25 mls), (solution B), was prepared. A 13.5ml volume of solution A was slowly added to
10 15ml of solution B under nitrogen atmosphere. The solution was stirred for five minutes, and the ethanol removed *in vacuo*, and the white solid repeatedly evaporated with benzene until dry. The resulting disodium salt of mercaptoleucine was dissolved in ~1ml of dimethylsulfoxide (DMSO) to which
15 was added 1.58g of compound 2 dissolved in 2mls of DMSO, and stirred overnight. The reaction mixture was combined with 175 ml of distilled water and extracted with 20ml portions of ether three times and then acidified with 5N HCl with stirring at 0°C. The aqueous solution was re-extracted 3X
20 with ethyl acetate. The extract was washed with a saturated NaCl solution and dried over sodium sulfate, filtered, and the ethyl acetate removed *in vacuo* yielding 1.05g of a clear oil. This was crystallized from ethyl acetate and hexane; yielding a white solid; (0.83g), (mp, 110-111), ([α]₂₅ = 52.5
25 (C0.88 l, MeOH)).

S)-(S)-tert-Butyloxycarbonyl-PheΨ[CH₂S]Leu-resin(Compound 4)

The resins utilized in solid phase peptide synthesis are prepared such that the alpha carboxyl group of the C-
30 terminal amino acid residue is covalently attached to the resin matrix. Although many support resins are known in the field, peptide synthesis is generally conducted in a reaction vessel on an insoluble resin support, generally with a styrene-1%-divinylbenzene polymer. The carboxy-
35 terminal amino acid is often attached to the resin by a

special organic linker, however, direct attachment to the resin is well known in the field. For example, resins with suitable organic linkers are the 4-(oxymethyl)phenylacetamidomethyl (PAM) resin or the p-benzyloxybenzyl alcohol
5 (WANG) resin.

Compound 3 was attached to a methyl benzhydramine resin by activating compound 3 (converted to the active ester) with hydroxybenzotriazole in acetonitrile/dimethylacetamide and dicyclohexylcarbodiimide in acetonitrile.

10

[Phe₈Ψ[CH₂S]Leu₉]Litorin (Compound 5)

The solid phase peptide synthesis for elongation of the designated amino acid sequence was performed on an Applied Biosystems peptide synthesizer using standard methodology,
15 that of the manufacture, and that known by people skilled in the art.

The completed resin bound peptide was cleaved from the resin employing hydrogen fluoride at 0°C in the presence of anisole (ethanedithiol) for 1 hr. Following, removal of the
20 HF the resin was stirred and extracted with diethyl ether (2x30ml) and extracted with 30% acetic acid. Lyphilization afforded crude product. A portion of the product was purified on preparative reverse phase high performance liquid chromatography with a C18 Dynamax column employing a
25 mobile phase gradient elution (acetonitrile gradient; established from reservoirs of acetonitrile and 0.1 %TFA in water). Fractions of the principle peak were collected monitoring absorbance of the compound at A₂₁₄.

30 [Phe₈Ψ[CH₂S(CH₃)]Leu₉]Litorin (Compound 6)

A sample of the [Phe₈Ψ[CH₂S]Leu₉]Litorin (5 mg) was stirred in 5 ml of iodomethane for one hour and the iodomethane was removed via evaporation to give the sulfur
ylide. The resulting product was further purified on
35 preparative reverse phase high performance liquid

chromatography with a C18 Dynamax Column employing a mobile phase gradient (15 min acetonitrile gradient 5-15% at 40 ml/min; established from reservoirs of acetonitrile and 0.1% TFA in water].

5

EXAMPLE 2

[t-Butyloxycarbonyl)-L-Leucine N-Methoxy-N-methylamide:10 Compound (7).

Triethylamine (10ml) is added to a stirred solution of Boc-Leucine in dichloromethane. Then sequentially, carbonyl-diimidazole (10mmol) are added followed by ,N-dimethylhydroxyamine hydrochloride (11 mmol), and
15 triethylamine (11mmol). The reaction is monitored by TLC, was found to be completely within an hour. The mixture is diluted with dicloromethane (250ml) and washed successively with 1N HCl, and a saturated sodium chloride solution. The organic solution was dried with magnesium sulfate, and the
20 solvent evaporated to give the desired product (9.0 mmol yield).

(t-Butyloxycarbonyl)-L-Leucinal: Compound (8).

Lithium aluminum hydride (2.5 equiv.) is added to a
25 stirred solution of compound (7). Reduction is complete within 15-20 minutes. The mixture is hydrolyzed with a solution of potassium hydrogen sulfate in water. Ether is added and the aqueous phase is separated and extracted. The organic phases are combined, washed with 1N HCl saturated
30 sodium hydrogen carbonate and saturated sodium chloride, and dried with magnesium sulfate. The solvent is evaporated to leave the desired product

35

[Phe₈Ψ[CH₂N(CH₃)]Leu₉]Litorin: (Compound 9)

5 Compound (8) was reacted with TFA-H-Leuciny-l-p-methylamine resin (1% cross-linked) in 1% acetic acid in DMF for 3 hours using NaBH₃CN (25 equivalents). The reaction was found to be complete based on the Kaiser test.

10 The solid phase peptide synthesis for elongation of the amino acid sequence was performed on an Applied Biosystems peptide synthesizer using standard methodology, that of the manufacture, and that known by people skilled in the art.

15 The peptides obtained by this method gave the desired molecular ion peak by FAB-MS and had an amino acid analysis in accordance with the desired peptide. In this way the following peptides having the stated properties were prepared.

20 (ID#9) pGlu Gln Trp Ala Val Gly His PheΨ[CH₂S(CH₃)]Leu-NH₂
 MW 1084

25 (ID#8) pGlu Gln Trp Ala Val Gly His PheΨ[CH₂N(CH₃)]Leu-NH₂
 MW 1066 FAB-MS (MH⁺) 1057 t_R
 60% peptide content

EXAMPLE 4

30 BINDING TO THE BOMBESIN RECEPTOR AS DEMONSTRATED BY
 IODINATED GRP

35 The pancreata from one or more mice were pooled and homogenized in 50 mM HEPES (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 1 mM EDTA and protease inhibitors (1 µg/ml aprotinin, leupeptin, pepstatin; 4 µg/ml bacitracin,

antipain, bestatin; 100 μ M PMSF) at 4°C and centrifuged at 37,500 X g for 15 minutes. The pellet was resuspended in 50 mM HEPES (pH 7.4) containing 10 mM EDTA, 300 mM KCl, and protease inhibitors, and then incubated for 30 minutes at 4°C. The suspension was centrifuged as above and the pellet was washed two times in 50 mM HEPES (pH 7.4) containing 8 μ g/ml thiorphan and protease inhibitors, and again centrifuged. The tissue was then resuspended in incubation buffer (1 ml per 4 mg pancreas) and incubated for 15 minutes at room temperature, then 250 μ l were added to each assay tube to commence the assay. The assay tubes contained incubation buffer consisting of 50 mM HEPES (pH 7.4), 0.5% BSA, protease inhibitors, 2 mM $MnCl_2$, 8 μ g/ml thiorphan, 1 μ M somatostatin, and concentrations of ^{125}I -GRP and peptides as needed in a final volume of 500 μ l. The assay was allowed to proceed to equilibrium for 90 minutes at room temperature. After this time, the contents of each tube was rapidly filtered over Whatman GF/B filters presoaked in 0.1% polyethyleneimine and the filters were rapidly washed three times with ice-cold 50 mM HEPES (pH 7.4). Filter-bound radioactivity was quantitated in a gamma counter. Competition of iodinated GRP binding by test compounds or standards was expressed as a percentage of ^{125}I -GRP binding in the absence of peptide. Affinity and maximal binding were calculated with LIGAND (Biosoft, Cambridge, UK) (Figures 1 and Figure 2)

30

35

EXAMPLE 5EFFECT OF ANALOGS ON THE BOMBESIN RECEPTOR AS DEMONSTRATED
BY PHOSPHATIDYLINOSITOL TURNOVER

5 Pancreata from mice were chopped at 350 μ m with a tissue
chopper and pooled. The chopped tissue was washed twice
with oxygenated Krebs-Hepes, then incubated for 30 minutes
in 37°C oxygenated Krebs-Hepes buffer with fresh buffer
after 15 minutes. The tissue was then incubated in this
10 buffer containing 200 μ Ci of [3 H] inositol at 37°C for 1
hour. The tissue was then washed twice and incubated for
another 30 minutes in oxygenated Krebs-Hepes (containing 10
mM Li $^+$) at 37°C with a fresh buffer change after 15 minutes.
Portions of the tissue mass (approximately 10 mg per assay
15 tube) were then placed in Li $^+$ buffer with protease
inhibitors, (40 μ g/ml bacitracin, 4 μ g/ml leupeptin, 4 μ g/ml
chymostatin, 8 μ g/ml thiorphan), 0.1% BSA, and 0.1-10 μ M
peptide in a final volume of 250 μ l. After 60 minutes at
room temperature, the phosphatidylinositol turnover was
20 terminated by the addition of 940 μ l chloroform:methanol
(1:2), followed by 310 μ l chloroform, followed by 310 μ l
water. Each tube was then vortexed for 5 seconds and then
centrifuged at 2500 x g for 10 minutes to separate the
phases. 50 μ l of the bottom phase (chloroform) was
25 withdrawn from each tube and placed in a counting vial,
dried, and counted in scintillation fluid. 900 μ l of the
top (aqueous) phase were then mixed with 2.1 ml water and
loaded onto a 0.5 ml Biorad AG-1X8 (formate) ion exchange
column. The material on the columns was washed in order
30 with: 1) 10 ml of water 2) 5 ml of 5 mM disodium
tetraborate/60 mM sodium formate 3) 10 ml of 1 M ammonium
formate in 0.1 M formic acid. The final (third) wash was
collected and one ml was mixed with 14 ml of Bio-Safe
scintillant and counted. The ratio of these counts (total
35

inositol phosphates) to the corresponding organic phase counts (inositol incorporated into the tissue) was then calculated for each sample. The ratios in the presence of test compound and/or standards were then compared to the ratios for control tubes (i.e., no stimulating agonist). The abilities of test compounds to stimulate phosphatidyl-inositol turnover were determined with the aid of a computer program.

EXPLANATION OF FIGURES

10

Figure 1 illustrates that ^{125}I -GRP binds to a single class of sites--the bombesin/GRP receptor--on murine pancreatic membranes (Example 1). Binding of 25-1600 pM ^{125}I -GRP was assayed in triplicate, then analyzed and plotted with LIGAND. The best computer fit of these data is 19 pM receptor per sample (~100 fmol receptor per mg membrane protein) with a K_d of 47 pM. The abscissa (x-axis) indicates the concentration of ^{125}I -GRP bound to the receptor. The ordinate (y-axis) indicates the concentration of ^{125}I -GRP bound to the receptor divided by the concentration of ^{125}I -GRP that is free (not bound). The straight line is indicative of a single class of sites; that is, ^{125}I -GRP binds to each of its receptors with the same affinity. Other experiments using 25-3200 pM ^{125}I -GRP or 10 pM ^{125}I -GRP \pm 8-500 pM GRP also indicated a similar K_d . This shows that binding to the receptor for bombesin/GRP can be measured with ^{125}I -GRP and murine pancreatic membranes.

Figure 2 illustrates the ability of bombesin analogs to bind to the GRP receptor as demonstrated by the ability of these peptides to compete for binding of ^{125}I -GRP to murine pancreatic membranes (Example 1). The abscissa (x-axis) logarithmically indicates the concentration of agonists being tested. The ordinate (y-axis) indicates the observed

35

binding for each tested peptide measured as a percentage of maximal ^{125}I -GRP binding (no peptide present). Binding of litorin (Δ) was assayed in triplicate at the indicated concentrations in the presence of 40 pM ^{125}I -GRP. Litorin 5 binding best fit a single class of sites with a $K_d=0.1$ nM. Binding of $(\text{Phe}^8\psi[\text{CH}_2\text{N}(\text{CH}_3)]\text{Leu}^9)\text{litorin}(\text{R})$ was assayed in duplicate at the indicated concentrations in the presence of 20 pM ^{125}I -GRP. Two such curves were co-analyzed with ^{125}I -GRP saturation curve (not shown) similar to that in Fig. 1: 10 all three curves were from the same experiment. $(\text{Phe}^8\psi[\text{CH}_2\text{N}(\text{CH}_3)]\text{Leu}^9)\text{litorin}$ binding best fits two classes of sites ($K_d=0.08$ and 16 nM) with 20% of the receptors in the high affinity state. Analysis of several other litorin and $(\text{Phe}^8\psi[\text{CH}_2\text{N}(\text{CH}_3)]\text{Leu}^9)\text{litorin}$ experiments have produced 15 similar results.

Figure 3 illustrates the ability of GRP and $(\text{Phe}^8\psi[\text{CH}_2\text{N}(\text{CH}_3)]\text{Leu}^9)\text{litorin}(\text{NMe})$, to stimulate phosphatidylinositol (PI) turnover in a dose-dependent 20 manner (Example 2). The ordinate (y-axis) indicates the observed PI turnover as a percentage of control. Values are mean \pm standard error of triplicate determinations. PI turnover by $(\text{Phe}^8\psi[\text{CH}_2\text{N}(\text{CH}_3)]\text{Leu}^9)\text{litorin}$ at concentrations indicated demonstrate that administration of the peptide 25 results in a statistically significant ($P<0.005$) increase in PI turnover.

Table I correlates abbreviated biological and chemical nomenclature, sequences, and sequence identification numbers 30 (ID#).

Table II compares the results of the earlier experiments (figures 1-2) for receptor affinity (K_d) and PI turnover for the bombesin analogs.

35

TABLE 1
Sequence of Peptides and Peptide Analogs

Sequence	I.D.#
Gastrin Releasing Peptide (14-27)	1
Bombesin	2
Litorin	3
[Leu ¹³ ψ[CH ₂ NH][Leu ¹⁴]bombesin	4
[Phe ¹³ ψ[CH ₂ S][Leu ¹⁴]bombesin	5
[Phe ⁸ ψ[CH ₂ S][Leu ⁹]litorin	6
[Phe ⁸ ψ[CH ₂ S(O)][Leu ⁹]litorin	7
[Phe ⁸ ψ[CH ₂ N(CH ₃)]Leu ⁹]litorin	8
[Phe ⁸ ψ[CH ₂ S(CH ₃)]Leu ⁹ -litorin	9
Acetyl[D-Ala ¹¹]bombesin(7-13)amide	10
Octyl[D-Ala ¹¹]bombesin(7-13)amide	11
Lauryl[D-Ala ¹¹]bombesin(7-13)amide	12
Palmityl[D-Ala ¹¹]bombesin(7-13)amide	13
Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH ₂	1
pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH ₂	2
pGlu-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH ₂	3
pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-ψ[CH ₂ N]Leu-NH ₂	4
pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Phe-ψ[CH ₂ S]Leu-NH ₂	5
pGlu-Gln-Trp-Ala-Val-Gly-His-Phe-ψ[CH ₂ S]Leu-NH ₂	6
pGlu-Gln-Trp-Ala-Val-Gly-His-Phe-ψ[CH ₂ S(O)]Leu-NH ₂	7
pGlu-Gln-Trp-Ala-Val-Gly-His-Phe-ψ[CH ₂ N(CH ₃)]Leu-NH ₂	8
pGlu-Gln-Trp-Ala-Val-Gly-His-Phe-ψ[CH ₂ S(CH ₃)]Leu-NH ₂	9
Nα-acetyl-Gln-Trp-Ala-Val-D-Ala-His-Leu-NH ₂	10
Nα-octyl-Gln-Trp-Ala-Val-D-Ala-His-Leu-NH ₂	11
Nα-lauryl-Gln-Trp-Ala-Val-D-Ala-His-Leu-NH ₂	12
Nα-palmityl-Gln-Trp-Ala-Val-D-Ala-His-Leu-NH ₂	13

TABLE II

Comparison of affinities of bombesin and litorin analogs and their effect on PI turnover.

Analog	Kd(nM)	Agonist ^b	PI Turnover %Inhibition ^c	Sequence I.D. #
Gastrin Releasing Peptide	0.02	+	ND	1
Bombesin	0.15	+	ND	2
Litorin	0.075	+	ND	3
[Leu ¹³ ψ[CH ₂ NH]Leu ¹⁴]bombesin	8.0	-	30*	4
[Phe ¹³ ψ[CH ₂ S]Leu ¹⁴]bombesin	2.8	-	54**	5
[Phe ⁸ ψ[CH ₂ S]Leu ⁹]litorin	3.4	-	42*	6
[Phe ⁸ ψ[CH ₂ S(O)]Leu ⁹]litorin I	1.8	-	53**	7a
[Phe ⁸ ψ[CH ₂ S(O)]Leu ⁹]litorin II	1.0	-	83**	7b
[Phe ⁸ ψ[CH ₂ N(CH ₃)]Leu ⁹]litorin	.019 & 23	-	-16	8
Phe ⁸ ψ[CH ₂ S(CH ₃)]Leu ⁹]litorin	ND	+	ND	9
Acetyl[D-Ala ¹¹]bombesin(7-13)amide	69	ND	24	10
Octanoyl[D-Ala ¹¹]bombesin(7-13)amide	5.0	-	82**	11
Lauryl[D-Ala ¹¹]bombesin(7-13)amide	320	-	53**	12
Palmityl[D-Ala ¹¹]bombesin(7-13)amide	350	-	45*	13

-41-

^aThe peptides listed were tested in both a competitive binding and PI-turnover assay in mouse pancreas as described in Examples 4 and 5; Kd values are averages of multiple experiments.

^b(+) represents agonist activity, (-) indicates no agonist activity, (ND) indicates not determined.

^cFor antagonist activity, % inhibition is referenced to the stimulation produced by 100 nM GRP. ND indicates not determined. Data for antagonists by the reduced bond analogs and the N-acyl analogs are from two different experiments with similar levels of GRP stimulation. *p < 0.05; **p < 0.01; ***p < 0.001.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Edwards, Vince
Fanger, Brad
- (ii) TITLE OF INVENTION: Bombesin Analogs
- (iii) NUMBER OF SEQUENCES: 13
- 10 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Marion Merrell Dow Inc.
(B) STREET: 2110 East Galbraith Rd.
(C) CITY: Cincinnati P. O. Box 156300
(D) STATE: Ohio
(E) COUNTRY: USA
(F) ZIP: 45215-6300
- 15 (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- 20 (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US M01598
(B) FILING DATE: 23-MAY-1991
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Collier, Kenneth J
(B) REGISTRATION NUMBER: P-34,982
(C) REFERENCE/DOCKET NUMBER: M01598 US
- 25 (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (513) 948-7834
(B) TELEFAX: (513) 948-7961

(2) INFORMATION FOR SEQ ID NO:1:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

35

(ix) FEATURE:

(A) NAME/KEY: Peptide

(B) LOCATION: 1..14

(D) OTHER INFORMATION: /note= "Gastrin Releasing Peptide
amino acids 14-27"

5

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 14

(D) OTHER INFORMATION: /note= "Xaa is Methionin-1-amide
(Met-NH2)"

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met	Tyr	Pro	Arg	Gly	Asn	His	Trp	Ala	Val	Gly	His	Leu	Xaa
1				5						10			

(2) INFORMATION FOR SEQ ID NO:2:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

20

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "Xaa is pyroglutamyl (pGlu)"

(ix) FEATURE:

25

(A) NAME/KEY: Modified-site

(B) LOCATION: 14

(D) OTHER INFORMATION: /note= "Xaa is Methionin-1-amide
(Met-NH2)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

30

Xaa	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Xaa
1				5						10			

35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Xaa is pyroglutamyl (pGlu)"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 9
- (D) OTHER INFORMATION: /note= "Xaa is Methionin-1-amide (Met-NH2)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Xaa Gln Trp Ala Val Gly His Phe Xaa
1 5

20 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Xaa is pyroglutamyl (pGlu)"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 13
- (D) OTHER INFORMATION: /note= "Xaa is a Leucine analog having a 1-methylene group, in place of a 1-carbonyl group, bonded to the alpha"

- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 13
 (D) OTHER INFORMATION: /note= "(cont'd) nitrogen of the subsequent amino acid"
- 5 (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 14
 (D) OTHER INFORMATION: /note= "Xaa is Leucin-1-amide (Leu-NH2)"
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

 Xaa Gln Arg Leu Gly Asn Gln Trp Ala Val Gly His Xaa Xaa
 1 5 10
- (2) INFORMATION FOR SEQ ID NO:5:
- 15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 14 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- 20 (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "Xaa is pyroglutamyl (pGlu)"
- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 13
25 (D) OTHER INFORMATION: /note= "Xaa is a Phenylalanine analog having a 1-methylene group, in place of a 1-carbonyl group, bonded to the alpha"
- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 13
30 (D) OTHER INFORMATION: /note= "(cont'd) nitrogen of the subsequent amino acid"
- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 14
35 (D) OTHER INFORMATION: /note= "Xaa is 2-thio-4-methylpent-1-amide ([S]Leu-NH2)"

-46-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Xaa Gln Arg Leu Gly Asn Gln Trp Ala Val Gly His Xaa Xaa
1 5 10

5 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Xaa is pyroglutamyl (pGlu)"

15 (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /note= "Xaa is a Phenylalanine analog having a 1-methylene group, in place of a 1-carbonyl group, bonded to "

20 (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /note= "(cont'd) the alpha nitrogen of the subsequent amino acid"

(ix) FEATURE:

- 25
- (A) NAME/KEY: Modified-site
 - (B) LOCATION: 9
 - (D) OTHER INFORMATION: /note= "Xaa is 2-thio-4-methylpent-1-amide ([S]Leu-NH2)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

30 Xaa Gln Trp Ala Val Gly His Xaa Xaa
1 5

35

-47-

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- 10 (A) NAME/KEY: Modified-site
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Xaa is pyroglutamyl (pGlu)"

(ix) FEATURE:

- 15 (A) NAME/KEY: Modified-site
(B) LOCATION: 8
(D) OTHER INFORMATION: /note= "Xaa is a Phenylalanine
analog having a 1-methylene group, in place of a
1-carbonyl group, bonded to the alpha"

(ix) FEATURE:

- 20 (A) NAME/KEY: Modified-site
(B) LOCATION: 8
(D) OTHER INFORMATION: /note= "(cont'd) nitrogen of the
subsequent amino acid"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "Xaa is
2-sulfoxide-4-methylpent-1-amide ([S]Leu-NH2)"

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Xaa Gln Trp Ala Val Gly His Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:8:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35

-48-

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "Xaa is pyroglutamyl (pGlu)"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 8

(D) OTHER INFORMATION: /note= "Xaa is a Phenylalanine analog having a 1-methylene group, in place of a 1-carbonyl group, bonded to the alpha"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 8

(D) OTHER INFORMATION: /note= "(cont'd) nitrogen of the subsequent amino acid"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 9

(D) OTHER INFORMATION: /note= "Xaa is N-methyl-leucin-1-amide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Xaa Gln Trp Ala Val Gly His Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 1

(D) OTHER INFORMATION: /note= "Xaa is pyroglutamyl (pGlu)"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 8

(D) OTHER INFORMATION: /note= "Xaa is Phenylalanine analog having a 1-methylene group, in place of a 1-carbonyl group, bonded to the"

-49-

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /note= "(cont'd) alpha nitrogen of the subsequent amino acid"

5

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 9
- (D) OTHER INFORMATION: /note= "Xaa is 2-thiomethyl-4-methylpent-1-amide ([S(CH3)]Leu-NH2)"

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Xaa Gln Trp Ala Val Gly His Xaa Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:10:

15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

20

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Xaa is N-alpha-acetyl-glutamine (Ac-Gln)"

(ix) FEATURE:

25

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 5
- (D) OTHER INFORMATION: /note= "Xaa is D-alanine (D-Ala or ala)"

(ix) FEATURE:

30

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 7
- (D) OTHER INFORMATION: /note= "Xaa is Leucin-1-amide (Leu-NH2)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

35

Xaa Trp Ala Val Xaa His Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Xaa is
N-alpha-octanoyl-glutamine (Oct-Gln)"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 5
- (D) OTHER INFORMATION: /note= "Xaa is D-alanine (D-Ala or
ala)"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 7
- (D) OTHER INFORMATION: /note= "Xaa is Leucin-1-amide
(Leu-NH2)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Xaa Trp Ala Val Xaa His Xaa
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "Xaa is
N-alpha-lauryl-glutamine"

-51-

- (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 5
 (D) OTHER INFORMATION: /note= "Xaa is D-alanine (D-Ala or ala)"
- 5 (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 7
 (D) OTHER INFORMATION: /note= "Xaa is Leucin-1-amide (Leu-NH2)"
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

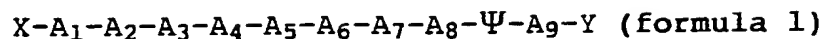
 Xaa Trp Ala Val Xaa His Xaa
 1 5
- (2) INFORMATION FOR SEQ ID NO:13:
- 15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- 20 (ix) FEATURE:
 (A) NAME/KEY: Modified-site
 (B) LOCATION: 1
 (D) OTHER INFORMATION: /note= "Xaa is N-alpha-palmityl-glutamine"
- (ix) FEATURE:
25 (A) NAME/KEY: Modified-site
 (B) LOCATION: 5
 (D) OTHER INFORMATION: /note= "Xaa is D-alanine (D-Ala or ala)"
- (ix) FEATURE:
30 (A) NAME/KEY: Modified-site
 (B) LOCATION: 7
 (D) OTHER INFORMATION: /note= "Xaa is Leucin-1-amide (Leu-NH2)"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

 Xaa Trp Ala Val Xaa His Xaa
 1 5
- 35

SUBSTITUTE SHEET

WHAT IS CLAIMED IS:

1. A peptide derivative of the formula



5

wherein;

- 10 X is an amino terminal residue selected from hydrogen, one or two alkyl groups from 1 to 16 carbon atoms, one or two acyl groups of from 2 to 16 carbon atoms, carbobenzyloxy or t-butyloxy carbonyl; unless the amino terminal amino acid is a cyclic derivative and thereby X is omitted.
- 15 A₁ is pGlu, Glu, or suitable acidic hydrophilic amino acid residue or is a sequence of 1 to 5 amino acids of Bombesin or a natural variants thereof, or a bond;
- A₂ is Gln, or suitable neutral amino acid residue;
- A₃ is Trp, or a suitable neutral or hydrophobic amino acid residue;
- 20 A₄ is Ala, or a suitable neutral or hydrophobic amino acid residue;
- A₅ is Val, or a suitable neutral or hydrophobic amino acid residue;
- A₆ is Gly, Ala, ala or a suitable neutral or hydrophobic amino acid residue;
- 25 A₇ is His, a suitable neutral or basic hydrophilic amino acid residue;
- A₈ is Phe, Leu, or is a suitable hydrophobic amino acid residue;
- 30 Ψ is a dipeptide determinant of A₈ Ψ A₉ wherein Ψ is [CH₂S(CH₃)] or [CH₂N(CH₃)], and wherein A₈ and A₉ designates the substituent amino acids;
- A₉ is Leu, Met, Nle, or is a suitable hydrophobic amino acid residue or is a sequence of 1 to 5 amino acid

35

residues of Bombesin or variants thereof, or a bond; and

- Y is a carboxy terminal substituent of the carbonyl group of the A₉ amino acid selected from OH, (C₁-C₈) alkoxyester, carboxamide, mono or di (C₁-C₈) alkyl amide, (C₁-C₈) alkylamine, (C₁-C₄) thioalkylether, or pharmaceutically acceptable salt thereof.

2. Claimed is a peptide of claim 1 wherein the peptide is pGlu-Gln-Trp-Ala-Val-Gly-His-PheΨ[CH₂N(CH₃)]Leu-NH₂.

3. Claimed is a peptide of claim 1 wherein the peptide is pGlu-Gln-Trp-Ala-Val-Gly-His-PheΨ[CH₂S(CH₃)]Leu-NH₂.

4. A peptide of one of Claims 1 or 3 which may be a pharmaceutically acceptable salt thereof or a pharmaceutical composition which utilizes a pharmaceutically acceptable carrier.

5. A pharmaceutical composition of a peptide of one of claims 1 or 3 for stimulating digestion in a patient in need thereof, which comprises administering to the patient an effective amount of the peptide.

6. Use in the manufacture of a medicament for decreasing food intake in a patient in need thereof which comprises administering to the patient an effective amount of a peptide derivative of one of claims 1 or 3.

7. Use in the manufacture of a medicament for stimulating growth of organ tissues of the lung, pancrease, or intestine in a patient in need thereof which comprises administering to the patient an effective amount of a peptide derivative of one of claims 1 or 3.

35

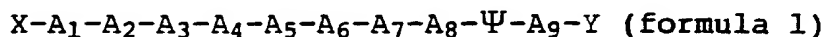
8. A method of temporarily stimulating growth of a tumor to increase susceptibility to chemotherapeutic agents which comprises administering to a patient in need thereof an effective amount of a peptide derivative of one of claims 1 or 2.

9. A method of stimulating natural killer cell activity against tumor cells by administering to a patient in need thereof an effective amount of a peptide derivative of one of claims 1 or 2.

10. A process for preparing a peptide derivative according to claims 1 or 2 or a pharmaceutically acceptable salt thereof comprising the steps of:

- a) using a resin with a suitably bound C-terminal protected dipeptide from the group $A_8\Psi A_9$, wherein Ψ is $[\text{CH}_2\text{S}(\text{CH}_3)]$ or $[\text{CH}_2\text{N}(\text{CH}_3)]$ and A_8 and A_9 designates the substituent amino acids;
- b) sequentially coupling the other alpha amino protected amino acids, A_7 through A_1 to achieve the protected amino acid sequence claimed; optionally having amino acid extension of the group C and N; and optionally having modification selected from species X and Y.
- c) removing said protecting groups;
- d) purifying the desired peptide.

11. A process for preparing a peptide derivative of the formula 1:



wherein;

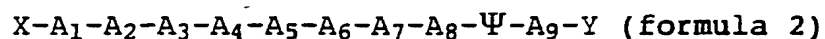
- 5 X is an amino terminal residue selected from hydrogen, one or two alkyl groups from 1 to 16 carbon atoms, one or two acyl groups of from 2 to 16 carbon atoms, carbobenzyloxy or t-butyloxy carbonyl; unless the amino terminal amino acid is a cyclic derivative and thereby X is omitted.
- 10 A₁ is pGlu, Glu, or suitable acidic hydrophilic amino acid residue or is a sequence of 1 to 5 amino acids of Bombesin or a natural variants thereof, or a bond;
- A₂ is Gln, or suitable neutral amino acid residue;
- A₃ is Trp, or a suitable neutral or hydrophobic amino acid residue;
- 15 A₄ is Ala, or a suitable neutral or hydrophobic amino acid residue;
- A₅ is Val, or a suitable neutral or hydrophobic amino acid residue;
- A₆ is Gly, Ala, ala or a suitable neutral or hydrophobic amino acid residue;
- 20 A₇ is His, a suitable neutral or basic hydrophilic amino acid residue;
- A₈ is Phe, Leu, or is a suitable hydrophobic amino acid residue;
- 25 Ψ is a dipeptide determinant of A₈ Ψ A₉ wherein Ψ is [CH₂S(CH₃)] or [CH₂N(CH₃)], and wherein A₈ and A₉ designates the substituent amino acids;
- A₉ is Leu, Met, Nle, or is a suitable hydrophobic amino acid residue or is a sequence of 1 to 5 amino acid residues of Bombesin or variants thereof, or a bond; and
- 30 Y is a carboxy terminal substituent of the carbonyl group of the A₉ amino acid selected from OH, (C₁-C₈) alkoxyester, carboxamide, mono or di (C₁-C₈) alkyl

35

amide, (C₁-C₈) alkylamine, (C₁-C₄) thioalkylether, or pharmaceutically acceptable salt thereof,

comprising methylating a peptide of the formula 2:

5



wherein, Ψ is [CH₂S] or [CH₂N],

10 with a iodomethane or suitable methylating reagent to form the compounds of formula 1.

15

20

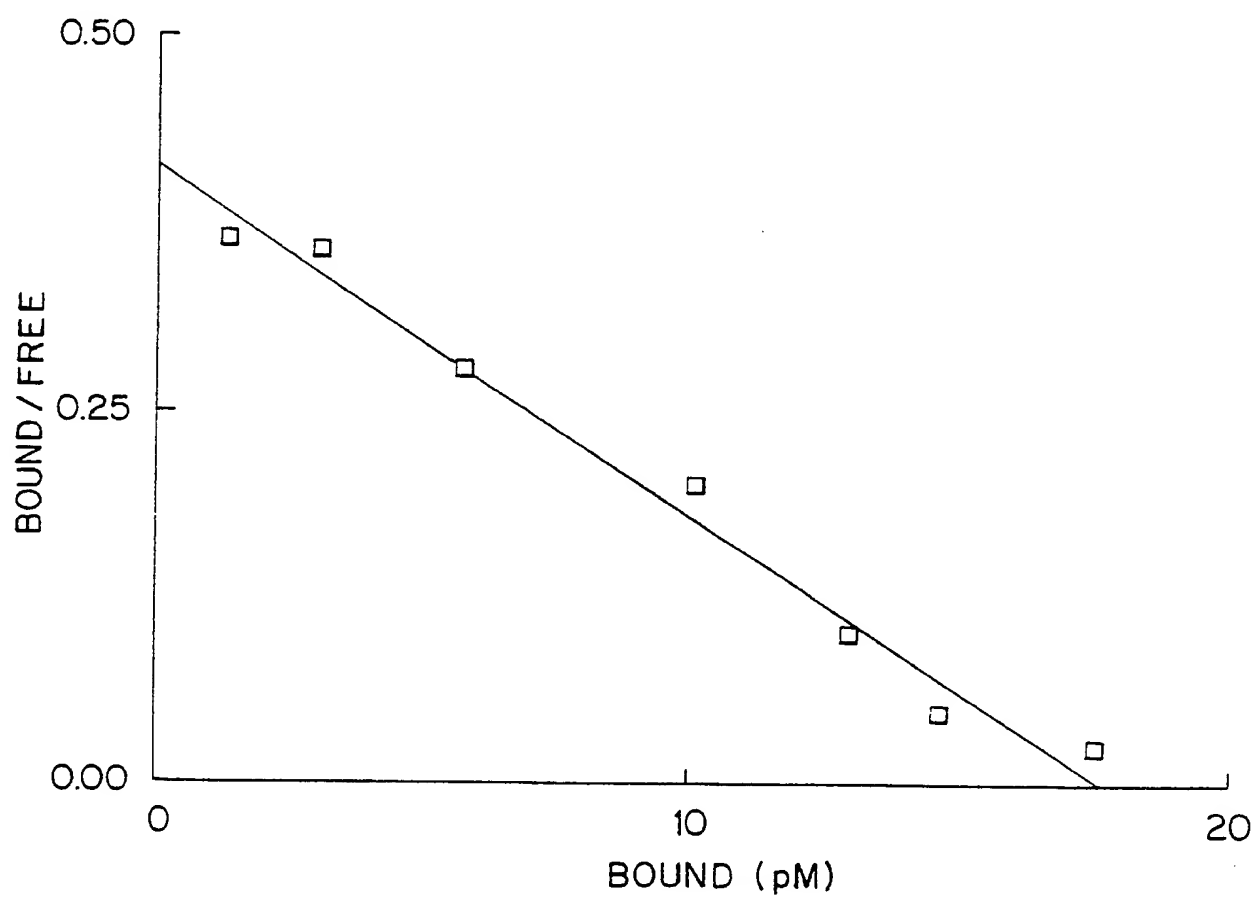
25

30

35

1 / 3

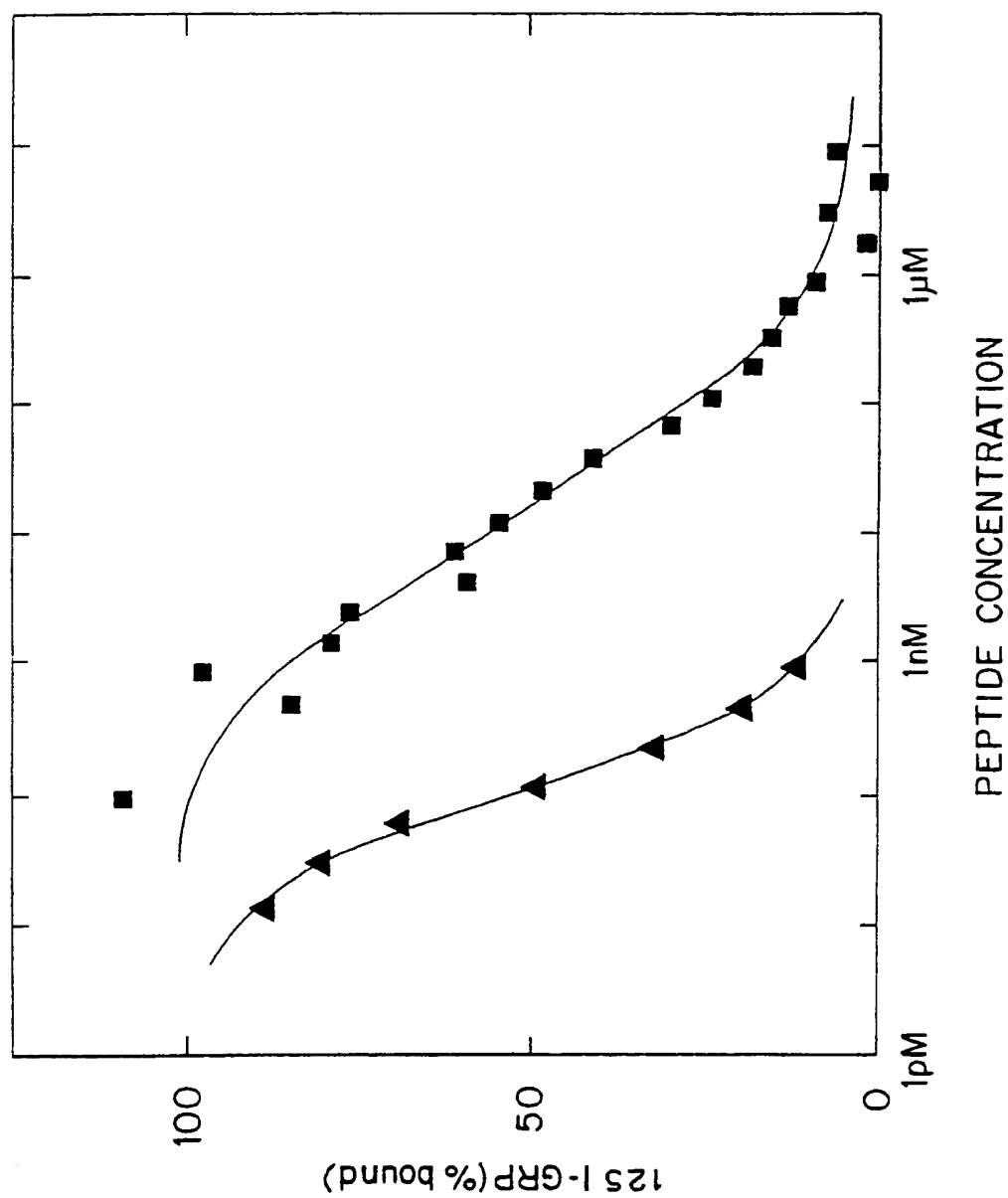
FIG. 1



SUBSTITUTE SHEET

2 / 3

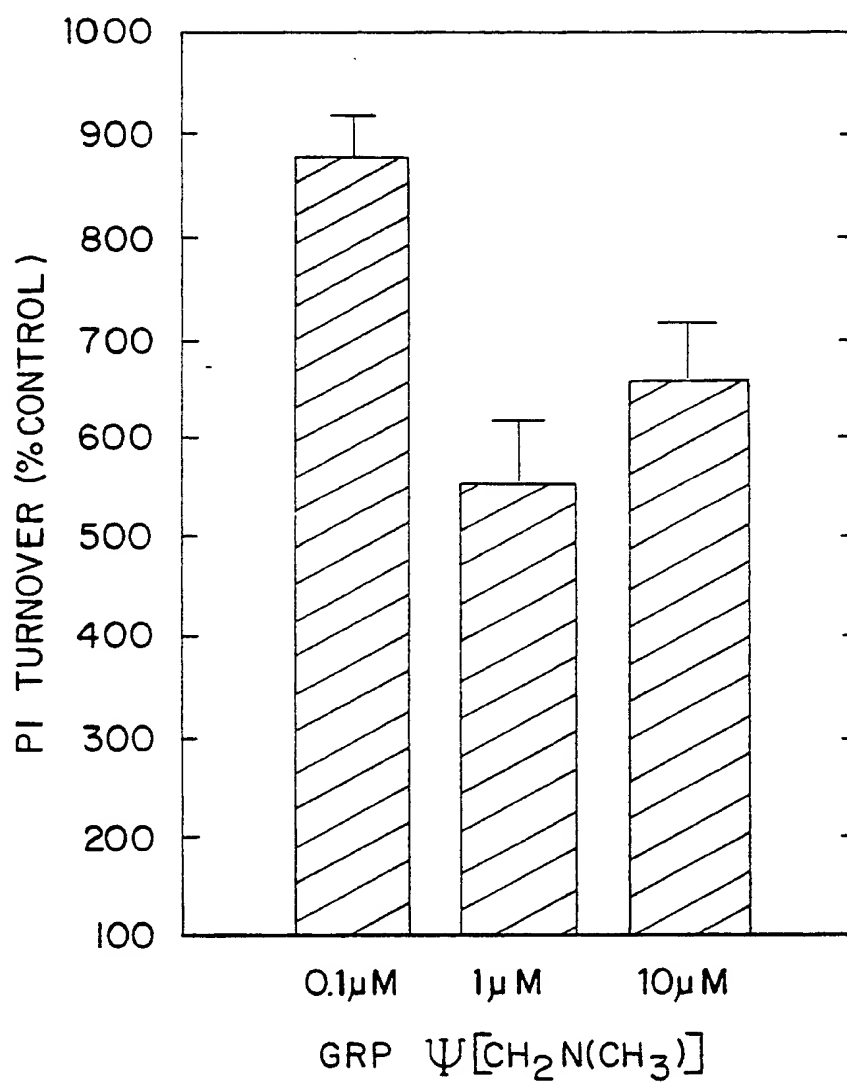
FIG. 2



SUBSTITUTE SHEET

3 / 3

FIG.3



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/03287

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K7/02; A61K37/02

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System

Classification Symbols

Int.Cl. 5

C07K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 264, no. 25, 1989, BALTIMORE, USA pages 14691 - 14697; COY ET AL: 'Short-chain pseudopeptide bombesin receptor antagonists with enhanced binding affinities for pancreatic acinar and Swiss 3T3 cells display strong antimitotic activity' * See page 14693, Table 1 *	1-11
A	EP,A,0 347 802 (MERRELL DOW PHARMACEUTICALS INC.) 27 December 1989 * See page 5, line 9 - page 6, line 14 * See page 8, line 19 - page 9, line 7 *	1-11

¹⁰ Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

25 AUGUST 1992

Date of Mailing of this International Search Report

01.09.92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

KORSNER S.E.

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 8-9 are directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the compounds.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9203287
SA 61321

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 25/08/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0347802	27-12-89	AU-B- 619857	06-02-92
		AU-A- 3652389	21-12-89
		JP-A- 2045497	15-02-90
